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DIA3 Phenotyping in Human Semen and Seminal Stains by Isoelectric Focusing

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Summary. The polymorphism of DIA3 was investigated by isoelectric focusing in semen samples from 235 unrelated Japanese volunteers and patients. Besides the three common phenotypes seven samples of the type 3-1 were observed. However, readable isoenzyme patterns were not demonstrated in semen samples of oligospermia under about 10×10^6/ml sperm cells. The allele frequencies were DIA3*1 = 0.821, DIA3*2 = 0.164, and DIA3*3 = 0.015. The DIA3*1 frequency in oligospermia (0.765) was lower than that in normospermia (0.836).

The isoelectric focusing method was successfully applied to phenotyping DIA3 in seminal stains; each phenotype was demonstrated at 37°C for up to 4 weeks, at room temperature for up to 8 weeks, and at 4°C for over 12 weeks after stain formation. In vaginal swabs the isoenzyme bands were very faint and not identifiable.

Key words: Enzyme polymorphism, DIA3 – Isoelectric focusing, DIA3 – Population study, Japanese – DIA3 phenotyping, in seminal stains

Zusammenfassung. Mittels isoelektrischer Fokussierung wurden die DIA3-Typen an Spermaproben von 235 nicht verwandten japanischen Spendern und Patienten untersucht. Neben den drei häufigen Phänotypen wurden sieben Proben des Typs 3-1 beobachtet. An Spermaproben von Oligospermie unter etwa 10×10^6/ml Spermien ließen sich jedoch keine ablesbaren Isoenzymbanden nachweisen. Die Allelfrequenzen betragen: DIA3*1 = 0,821, DIA3*2 = 0,164 und DIA3*3 = 0,015. Die DIA3*1-Frequenz bei Oligospermie (0,765) war niedriger als diejenige bei Normospermie (0,836).

Die Isoelektrofokussierungsmethode wurde mit Erfolg zur DIA3-Typisierung an Spermaspuren angewandt; jeder Phänotyp konnte bei 37°C bis zu 4 Wochen, bei Zimmertemperatur bis zu 8 Wochen und bei 4°C über 12 Wochen nach Beginn der Lagerung nachgewiesen werden. Bei Scheidenabstrichen waren die Isoenzymbanden sehr schwach und nicht erkennbar.

Schlüsselwörter: Enzympolymorphismus, DIA3 – Isoelektrofokussierung, DIA3 – Populationsstudie, Japaner – DIA3-Typisierung, an Spermaspuren

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Introduction

The genetic polymorphism of human sperm diaphorase was first discovered by Caldwell et al. (1976) and was later designated as DIA3 (Fisher et al. 1977). Using polyacrylamide or starch gel electrophoresis, they demonstrated three phenotypes DIA3 1, 2-1, and 2, which are controlled by two autosomal codominant alleles DIA3*1 and DIA3*2.

Kühnl et al. (1977) revealed the existence of the third common allele DIA3*3 by means of both agarose gel electrophoresis and isoelectric focusing. Moreover, they proposed the use of this polymorphic system for forensic trace investigations. Oepen et al. (1980) adopted the starch gel electrophoretic method recommended by Edwards et al. (1979) to separate DIA3 isoenzymes from dried seminal stains.

In the present study, the polymorphism of DIA3 in a Japanese population was investigated using an improved isoelectric focusing technique. Furthermore, this technique was applied to phenotyping DIA3 from seminal stains under various conditions of storage.

Materials and Methods

Semen. Ejaculates were obtained from 96 unrelated male volunteers living in a central part of Japan, Yamanashi Prefecture, and from 156 male patients at the Fertility Clinic, Yamanashi Prefectural Central Hospital. They consisted of 186 samples of normospermia (sperm counts over 40×10^6/ml) and 66 samples of oligospermia (sperm counts under 40×10^6/ml). The specimens were stored at −20°C until use.

Seminal Stains. Twenty semen samples of normospermia with known phenotypes were dropped on filter paper (Toyoroshi no. 2, Tokyo, Japan). They were stored in a thermostatic chamber at 37°C, at room temperature, and in a refrigerator at 4°C, and were examined weekly over a period of 12 weeks.

Vaginal Swabs. Vaginal swabs were collected at the Obstetrics and Gynecology Clinic, Yamanashi Prefectural Central Hospital, from 30 women who had an abstinence period over 7 days.

Isoelectric Focusing. Initial experiments were carried out by the method of Kühnl et al. (1977) using Ampholine, pH 3.5–10 (LKB, Bromma, Sweden). However, separation of the DIA3 isoenzymes was not always satisfactory, especially in the analysis of aged seminal stains. We therefore tried various modifications of the isoelectric conditions, in particular changes in the Ampholine intervals, and the procedure for mixing Ampholines of different pH intervals resulted in improved resolution of the isoenzyme components presumably due to an expanding effect of the pH gradient.

The gel plate ($230 \times 110 \times 0.5$ mm) was composed of 20 ml stock solution (5.25% acrylamide/0.25% N,N'-methylenebisacrylamide), 0.33 ml Ampholine, pH range 3.5–10 (LKB), 0.33 ml Ampholine, pH range 4–6 (LKB), 0.33 ml Ampholine, pH range 6–8 (LKB), 0.3 ml 0.01% riboflavin, and 2.5 g sucrose. The electrode paper strips were soaked with 1 *M* phosphoric acid for the anode and with 1 *M* sodium hydroxide for the cathode.

Semen samples were applied to the gel surface 2 cm from the anode using 5×6 mm filter paper (Toyoroshi no. 2). Seminal stains on filter paper were cut in 5×6 mm pieces. Vaginal swabs on cotton material were severed from the stick and trimmed into 5×6 mm pieces. The above pieces of stains were moistened with a minimum amount of distilled water just before analysis and directly applied to the gel plate.

Isoelectric focusing was started with an initial voltage of 200 V. The voltage was increased every 10 min to a limit of 1,400 V for 60 min. Focusing was then continued at a constant voltage of 1,400 V for 120 min. During focusing the gel plate was cooled by circulating water at 4°C.

Staining. After electrofocusing, the gel was stained essentially by the method of Hopkinson et al. (1970). The staining mixture consisted of 0.5 mg dichlorophenol-indophenol (Wako Chemicals, Osaka, Japan), 10 mg NADH (Oriental Yeast, Tokyo, Japan), 5 mg MTT (Sigma Chemical Co., St. Louis, MO, USA), 15 ml 0.05 *M* tris-HCl buffer (pH 8.5), and 15 ml 2% melted agar solution. The plate was covered with the agar reaction mixture and incubated in the dark at 37°C for 60 min. DIA isoenzymes appeared as dark purple bands on a pale background.

Results and Discussion

Distribution of DIA3 Types

The DIA3 isoenzyme patterns revealed by the present isoelectric focusing technique were nearly comparable to those reported by Kühnl et al. (1977), as illustrated in Fig. 1. The staining intensities of the isoenzyme components roughly depended on the number of sperm cells, and readable patterns were not demonstrated in 17 samples of oligospermia under about 10×10^6/ml sperm cells.

Table 1 shows the distribution of DIA3 types in 235 semen samples from Japanese males (186 samples of normospermia and 49 samples of oligospermia). The observed numbers were in good agreement with the numbers expected according to the Hardy-Weinberg equilibrium.

It is interesting to note that the DIA3*1 allele frequency in the samples of oligospermia (0.765) is significantly lower than that in the samples of normospermia (0.836), contrary to the expectation of Caldwell et al. (1976) or of Kühnl et al. (1977). We cannot find any reason to explain such a difference. This might be ascribable to the relatively small size of the sample population for oligospermia.

Table 2 lists the allele frequencies of DIA3 types in various racial groups so far reported. The DIA3*1 allele frequency in our sample is almost similar to

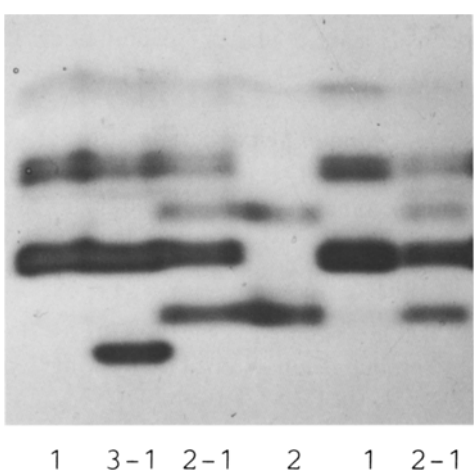

Fig. 1. Isoelectric focusing patterns of DIA3 types in semen. The anode is at the *top*

Table 1. Distribution of sperm DIA3 types in Japanese

Phenotype	No. observed (%)			No. expected
	Normo-spermia[a]	Oligo-spermia[b]	Total[c]	
1	129	27	156 (66.4)	158.4
2-1	47	20	67 (28.5)	63.3
2	4	1	5 (2.1)	6.3
3-1	6	1	7 (3.0)	5.8
3-2	0	0	0 (0.0)	1.2
3	0	0	0 (0.0)	0.1
Total	186	49	235 (100.0)	235.1

[a] DIA3*1 = 0.836, DIA3*2 = 0.148, DIA3*3 = 0.016; $\chi^2 = 1.132$, $df = 3$, $0.8 > P > 0.7$
[b] DIA3*1 = 0.765, DIA3*2 = 0.225, DIA3*3 = 0.010; $\chi^2 = 1.889$, $df = 3$, $0.7 > P > 0.5$
[c] DIA3*1 = 0.821, DIA3*2 = 0.164, DIA3*3 = 0.015; $\chi^2 = 2.068$, $df = 3$, $0.7 > P > 0.5$

Table 2. Allele frequencies of DIA3 types in various racial groups

Population	No. observed	Allele frequency			References
		DIA3*1	DIA3*2	DIA3*3	
American	52	0.71	0.29		Caldwell et al. (1976)
German	141	0.7553	0.2234	0.0213	Kühnl et al. (1977)
German	262	0.8034	0.1966		Kopetz et al. (1979)
English	145	0.80	0.20		Fisher et al. (1977)
English	346	0.762	0.227	0.012	Edwards et al. (1979)
Japanese	51	0.84	0.16		Suyama et al. (1979)
Japanese	263	0.837	0.143	0.020	Sebetan et al. (1982)
Japanese	235	0.821	0.164	0.015	Present study

that in other Japanese subpopulations (Suyama et al. 1979; Sebetan et al. 1982), but considerably higher than that in Americans (Caldwell et al. 1976) or Europeans (Fisher et al. 1977; Kühnl et al. 1977; Edwards et al. 1979; Kopetz et al. 1979).

Phenotyping in Seminal Stains

By the present technique fairly clear DIA3 patterns were obtained also from dried seminal stains though the bands became fainter and more indistinct with increasing time of storage. Figure 2 shows the isoelectric focusing patterns of DIA3 types in seminal stains stored at room temperature for 2 weeks and for 4 weeks.

Table 3 summarizes the results for the determination limits of DIA3 types in 20 seminal stains stored at varying temperatures. Our samples included 11 DIA3 1, six DIA3 2-1, one DIA3 2, and two DIA3 3-1. All the seminal stains examined were typed for DIA3 at 37°C for periods of up to 4 weeks, at room temperature for periods of up to 8 weeks, and at 4°C even for periods of more

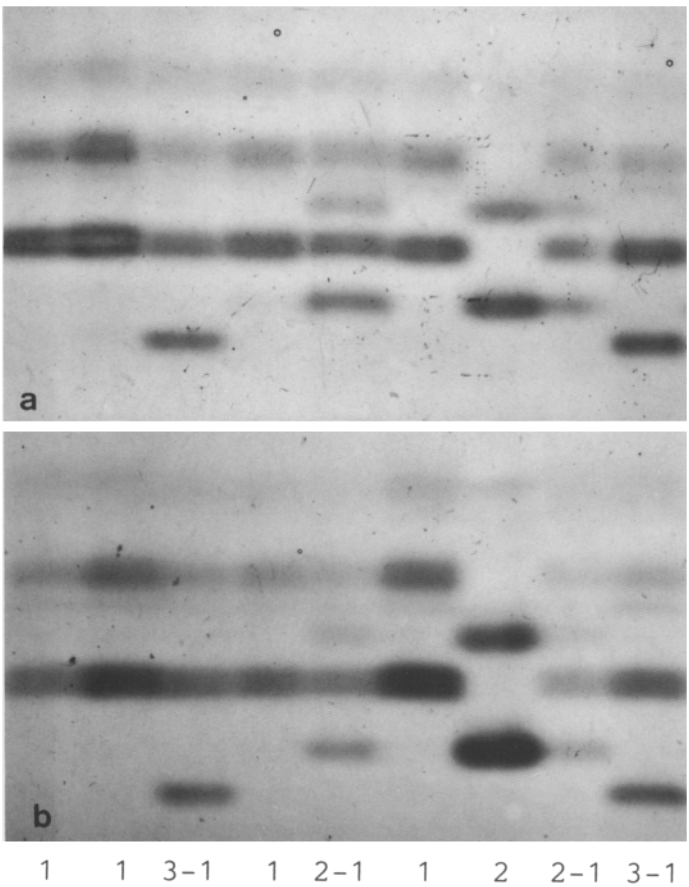

Fig. 2a, b. Isoelectric focusing patterns of DIA3 types in seminal stains stored at room temperature for 2 weeks (**a**) and for 4 weeks (**b**). The anode is at the *top*

than 12 weeks. No significant differences on the determination limits were observed among the four phenotypes.

The modified isoelectric focusing method described here allows DIA3 phenotyping for at least 2 months at room temperature. This time limit of determination is apparently superior to that obtained by starch gel electrophoretic experiments of 4 weeks (Oepen et al. 1980). The DIA3 phenotyping by isoelectric focusing would provide a powerful means for the grouping of seminal stains in forensic casework investigations. However, it must be taken into account that in case of azoospermia, extreme oligospermia or vasectomy the DIA3 isoenzymes cannot be detected from seminal stains.

Phenotyping in Vaginal Swabs

In view of the fact that DIA3 occurs also in adult ovary, oviduct, and uterus (Kühnl et al. 1977), phenotyping of DIA3 was attempted on vaginal secretions

Table 3. Positive results for the determination limits of DIA3 types in 20 seminal stains stored at 37°C, room temperature, and 4°C

Phenotype	No. tested	Temperature	Age of seminal stains (weeks)											
			1	2	3	4	5	6	7	8	9	10	11	12
1	11	37°C	11	11	11	11	10	6	5	4	4	3	3	1
2-1	6		6	6	6	6	4	4	4	3	3	2	2	1
2	1		1	1	1	1	1	1	1	1	1	1	1	1
3-1	2		2	2	2	2	2	2	2	1	1	1	1	0
1	11	Room temperature	11	11	11	11	11	11	11	11	11	9	9	7
2-1	6		6	6	6	6	6	6	6	6	5	4	4	4
2	1		1	1	1	1	1	1	1	1	1	1	1	1
3-1	2		2	2	2	2	2	2	2	2	2	2	2	1
1	11	4°C	11	11	11	11	11	11	11	11	11	11	11	11
2-1	6		6	6	6	6	6	6	6	6	6	6	6	6
2	1		1	1	1	1	1	1	1	1	1	1	1	1
3-1	2		2	2	2	2	2	2	2	2	2	2	2	2

from the female reproductive tracts. Activity was detected in 12 of 30 samples of vaginal swabs examined, but the patterns were very faint and not identifiable.

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